PHYLOGENETIC RELATIONSHIPS BETWEEN LUBOMIRSKIIDAE, SPONGILLIDAE AND SOME MARINE SPONGES ACCORDING PARTIAL SEQUENCES OF 18S rDNA

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Two families of Porifera are represented in Lake Baikal, Russia: cosmopolitan Spongillidae and endemic Lubomirskiidae. Systematics and phylogeny of Lubomirskiidae are still poorly known. Indeed, there is little agreement on the origin of freshwater sponges in general, and this group is considered to be polyphyletic. Latest morphological and embryological data indicate that Lubomirskiidae and Spongillidae are closely related. Using molecular data we explored the possible origins of Lubomirskiidae and determined the closest relatives of Spongillidae and Lubomirskiidae among marine sponges. Partial sequences of 18S rDNA for *Halichondria japonica*, *Lubomirskia abietina*, *Swartschewskia papyracea*, *Spongilla lacustris* and *Ephydatia muelleri* were compared with available sequences of 18S rDNA of other Porifera from the GenBank. Parsimony and neighbour-joining analyses gave trees of similar topology. Molecular data were in accordance with the notion of close relationships of endemic and cosmopolitan families. Some marine sponge families are assumed to be related to freshwater sponges.❐ *Porifera, Lake Baikal, Spongillidae, Lubomirskiidae, 18S rRNA, phylogeny, freshwater sponges.*

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Three families of Porifera inhabit freshwater: Spongillidae, Lubomirskiidae and Potamolepidae. The problem of the origin of endemic and cosmopolitan freshwater sponges, their relationships with each other and with marine sponges, repeatedly attract the attention of scientists. Marshall (1885) suggested freshwater sponges were polyphyletic, with Renieridae (Haplosclerida) possibly being their closest marine relative. The idea of a polyphyletic origin for freshwater sponges was subsequently discussed and emphasised by many authors. In describing the genus *Sterastrolepis*, believed to be a Neotropical representative of Potamolepidae, Volkmer-Ribeiro & De Rosa-Barbosa (1978) noted that the characteristics of its gemmoscleres were too different to assign this family to Haplosclerida. On the basis of gemmule structure, gemmosclere and skeleton peculiarities, they confirm Briens' (1970) assumption about the close relationship of Potamolepidae with Hadromerida. They also favour the hypothesis of a passive mechanism of invasion into freshwater habitats by marine sponges, noting that endemic freshwater genera (e.g. *Ochridaspongia*, *Pachydictium*, *Lubomirskia*) have been recorded from ancient lakes, remnants from past sea levels, but not from estuaries. Evidence for a hadromerid origin of some freshwater sponges (Volkmer-Ribeiro & Watanabe, 1983) is also provided by the Japanese sponge *Sanidastra yokotonensis*. Volkmer-Ribeiro (1990) also hypothesised that the Neotropical genus *Metania* may be related to the marine poecilosclerid genus *Acarnus*. Conversely, Racek & Harrison (1975), using palaeontologic data, suggest that *Spongillidae* was monophyletic having evolved from *Radiospongilla* stock.

The endemic family Lubomirskiidae, inhabiting Lake Baikal, has approximately 10 species belonging to 3 genera: *Lubomirskia*, *Baikalospongia* and *Swartschewskia* (Rezvoi, 1936). At present the systematics and phylogeny of this family is still poorly known. The history of study on the origin of Lubomirskiidae shows a number of contrary opinions. Dybowsky (1882), Swartschewsky (1902), Annandale (1913) and Rezvoi (1936) believed Lubomirskiidae was closely related to marine sponges and not to Spongillidae, owing to their considerable morphological differences. Later palaeontological studies (Martinson, 1940) hypothesised that

Lubomirskiidae were representatives of the mezolimnological fauna, originating mueh later than the usual palaeolimnological fauna to which Spongillidae belongs. In contrast to these beliefs, the latest comparative morphological data indicate a close relationship between Spongillidae and Lubomirskiidae (Efremova, 1981), supported by data on their loss of sexual reproduction by gemmules as an adaptive feature (Efremova, 1994). To solve contradictions in the systematic and phylogenetic interpretation of morphologieal data rDNA analysis is now widely used (e.g. Christen et al., 1991; Halanyeh, 1991). Although this method has been succesfully used for some marine sponge families (Lafay et al., 1992; West & Powers, 1993; Kelly-Borges & Pomponi, 1994) there are no previous studies on molecular phylogeny of freshwater sponges. In this study we apply partial 18S rDNA sequence analysis, firstly to explore the origin of Lubomirskiidae, and secondly to obtain new data on the origin of freshwater sponges in general.

TABLE 1. Classification of the species used in this study.

Classification	GenBank accession number	References
CNIDARIA: ANTHOZOA		
Parazoanthus axinella (Zoantharia: Zoantidae: Parazoanthida)	U42453	Cavalier-Smith, 1996
PORIFERA: DEMOSPONGIAE		
Axinella polypoides (Axinellida: Axinellidae)	U43190	Cavalier-Smith, 1996
Tetilla japonica (Spirophorida: Tetillidae)	D15067	Kobayashi et al., 1993
Microciona prolifera (Poecilosclerida: Microcionidae)	L10825	Wainright, 1993
Halichondria japonica (Halichondrida: Halichondridae)	AF058946	this study
Lubomirskia abietina (Haplosclerida: Lubomirskiidae)	AF058947	this study
Swartschewskia papyracea (Haplosclerida: Lubomirskiidae)	AF058948	this study
Ephydatia muelleri (Haplosclerida: Spongillidae)	AF058949	this study
Spongilla lacustris (Haplosclerida: Spongillidae)	AF058945	this study
PORIFERA: CALCAREA		
Clathrina cerebrum (Calcinia: Clathrinida: Clathrinidae)	U42452	Cavalier-Smith, 1996
Scypha ciliata (Calcaronia: Sycettida: Sycettidae)	L10827	Wainright, 1993
Scypha calcaravis (Calcaronia: Sycettida: Sycettidae)	D15066	Kobayashi et al., 1993

MATERIALS AND METHODS

Specimens of *Lubomirskia abietina*, *Swartschewskia papyracea*, *Spongilla lacustris* and *Ephydatia muelleri* were colleeted from Lake Baikal (Russia) and specimens of *Halichondria japonica* were eollected from Desaki seashore (Japan) by SCUBA diving in depths between 0.5-13.5m. All specimens were photographed alive. Data on ecology, habitat and texture were recorded. Part of eaeh sample was fixed in 70% ethanol for taxonomic identification, another part was frozen in liquid nitrogen for molecular analysis. Total genomie DNA extraction was performed with standard phenol method (Sambrook et al., 1989) and with CTAB method (Gustincieh et al., 1991). PCR primer design was performed by alignment of Porifera 18S rRNA sequences availablc from GenBank (see Table 1). As sponges harbour a large number of symbionts, in addition to universal primers, sponge-specific primers were also designed. The primers correspond to the V4 and V5 regions of 18S rRNA: R1 (5'-TAAAAAGCTCGTAGTTGGAT-3'; forward, universal, eorrespond to positions 629-647 in *Axinella polypoides* 18S rRNA (GenBank accession number U43190)); L1 (5'-GGACTACGACGGTATCTGAT-3'; reverse, universal (1008-1026)); R2 (5'-GTAGTGGC CTACCATGGTTGC-3'; forward, sponge-speeific (342-361)); L2 (5'-CTAATTTTTTCAAAG TAAACGTCCCGA-3'; reverse, sponge-specific (749- 777)).

The primers were synthesised by H-phosphonate method. Two overlapping fragments of the 18S rRNA gene (400bp eaeh) were amplified. A 25µl PCR reaction mix contained 2.5µl of 10×PCR Buffer (Promega), 3µl of $MgCl_2$ (25mM), 0.5µl of each primer (10pmol/µl), 1µl of dNTP mix (100mM eaeh), 1µl of DNA (~0,1µg), 0.2µl of Taq DNA polymerase, 25µl of ddH_2O. Cycle parameters were: initial denaturation at 94°C for 120secs, followed by 40 cyeles of denaturation at 94°C for 60secs, anneling at 45°C for 60secs, and extension at 72°C for 60secs, followed by a final extension of 8mins at 72°C. About 6 tubes of each PCR reaction were purified by electrophoresis in low melting agarose. PCR fragment purification was carried out twice with equal volume of phenol, followed by precipitation by 2 volume of ethanol and 0.1 volume of 10M ammonium aeetate and washing in 70% ethanol (Sambrook et al., 1989). PCR

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Lubomirskia   CGGGTGACGGAGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCAC
Swartshewskia ..........................................................
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  ..............A...........................................

Lubomirskia   CCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACTCGGGGAGGTAGTGACAA
Swartshewskia ..........................................................
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  ..........................................................

Lubomirskia   AATAACAATGCCGGGCTATCTTTAGTCTGGCAATTGGAATGAGAACAATGTAAATACC
Swartshewskia ..........................................................C...
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  ................-.G.....................T.....C.....C..

Lubomirskia   AACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
Swartshewskia ..........................................................
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  ..........................................................

Lubomirskia   AGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGGGCAGGAGG
Swartshewskia ..........................................................
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  ..............................................TG.CCT.

Lubomirskia   CGGTCCGCCGAAAGGTAGGTACTGGACGCCAGCCCTTTTTCTCGAAGGCCCCATCTGC
Swartshewskia ..........................................................
Spongilla     T.............C.........................G.....................
Ephydatia     ..........................................................
Halichondria  T.......---.....GA.......T.AG.C......CC........A.....GA....

Lubomirskia   TTCACTG-AGTGGTAGGGGAGTTCGGGACGTTTACTTTGAAAAAATTAGAGTGTTCAA
Swartshewskia .......-..................................................
Spongilla     ..T....-..................................................
Ephydatia     .......-..................................................
Halichondria  .......T......C...T.......................................

Lubomirskia   CAGGCCGTCGCTTGAATACGTTAGCATGGAATAATGGAATAGGACTTCGGTTCTATTT
Swartshewskia ..........................................................
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  .....TA....C........A.....................G.....C.....-......

Lubomirskia   TTGGTTTCTGGGACCGAAGTAATGATTAAGAGGGACAGTTGGGGGCATTCGTATTCAA
Swartshewskia ..........................................................
Spongilla     ..........................................................
Ephydatia     ..........................................................
Halichondria  .........A..G.....................................TT-

Lubomirskia   GTCAGAGGTGAAATTCTTGGATTTATGGAAGACGAACAACTGCGAAAGCATTTGCCAA
Swartshewskia .................C........................................
Spongilla     ................C.........................................
Ephydatia     ..........................................................
Halichondria  --.........................A.........T.G...................

Lubomirskia   ATGTTTTCATT
Swartshewskia ...........
Spongilla     ...........
Ephydatia     ...........
Halichondria  ...........
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FIG. 1. Alignment of partial 18S rDNA sequences (630 bp) obtained. Only nucleotides that differ from those of *Lubomirskia abietina* are indicated (identities are denoted by points and deletions by hyphens). GenBank accession numbers are: *Halichondria japonica* AF058946, *Lubomirskia abietina* AF058947, *Swartschewskia papyracea* AF058948, *Spongilla lacustris* AF058945 and *Ephydatia muelleri* AF058949.

fragments were sequenced on both strands using fmol DNA sequencing System (Promega) according to the published protocol. Cycle parameters were: initial denaturation at 95°C for 120secs, followed by 30 cycles of denaturation at 95°C for 30secs, annealing at 42°C for 30secs, and extension at 70°C for 60 secs. The structures obtained were aligned manually with the help of the GeneTools package (Rosenchuk, 1991). Neighbour-joining analysis was derived using Treecon for Windows (Van de Peer, 1994). The distance estimation was carried out using the formula of Kimura (1980). Bootstrap values were calculated from 100 replicates. *Parazoanthus axinellae* was used as the outgroup. Programs SEQBOOT, DNAPARS and CONSENSE of PHYLIP 3.5c package (Felsenstein, 1995) were used to construct maximum parsimony trees. Bootstrap analyses with 100 replications were carried out.

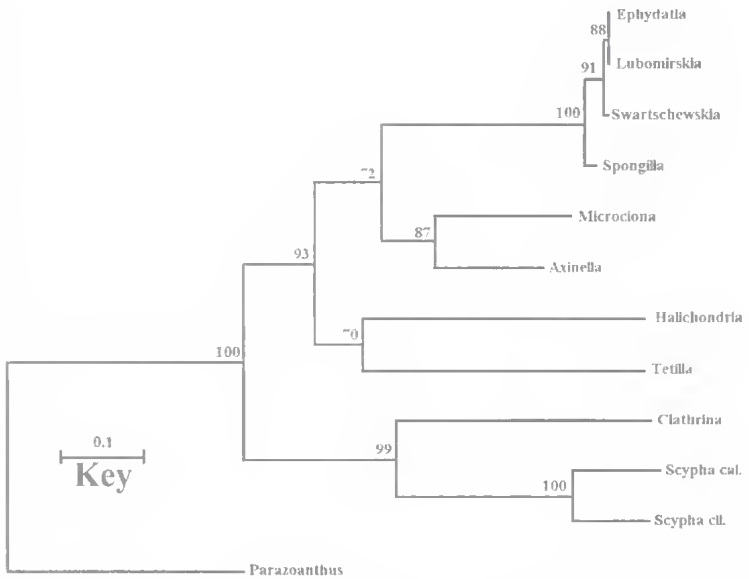

FIG. 2. Phylogenetic relationships of the Lubomirskiidae, Spongillidae and other Porifera based on neighbour-joining analysis of 18S rDNA (630bp). Bootstrap percentages are shown at the nodes for 100 resamplings. *Parazoanthus axinellae* used as the outgroup.

RESULTS AND DISCUSSION

We obtained partial 18S rRNA gene sequences (~630bp) for five species of Porifera. GenBank accession numbers are as follows: *Halichondria japonica* (AF058946), *Lubomirskia abietina* (AF058947), *Swartschewskia papyracea* (AF058948), *Spongilla lacustris* (AF058945) and *Ephydatia muelleri* (AF058949). Two specimens of each species were used to obtain sequences. All structures were aligned successfully, and common length of alignment was 630bp (Fig. 1).

There are a few nucleotide differences between 18S rDNA structures obtained for freshwater sponges compared to those from marine sponges. Sequences from the marine sponge *H. japonica* have many more transitions/ transversions events, and insertion/deletion events were observed only this species. *Lubomirskia* and *Ephydatia* show no nucleotide differences in their 18S rDNA sequences, indicating a very high level of genetic relationships between them.

To study the molecular relationships between freshwater and marine sponges, sequences from other Porifera available from GenBank (see Table 1) were included in the alignment.

Figure 2 shows a tree obtained by neighbour-joining analysis with *Parazoanthus axinellae* as the outgroup. High bootstrap values show that all clusters are statistically significant. *Spongilla, Lubomirskia, Swartschewskia* and *Ephydatia* form a common clade. A sister branch formed by *Axinella* and *Microciona* is the most closely situated to this clade. Parsimony analysis, performed on the basis of these sequences, provides a similar topology (not shown here). These data confirm that freshwater sponge genera form a closely related group and, except for *Axinella* and *Microciona*, *Halichondria* and *Tetilla*, also refer to the neighbouring cluster.

These molecular data are in accordance with the notion of a close relationship between endemic and cosmopolitan families. They do not support the idea that Lubomirskiidae has an independent origin from Spongillidae. These data also suggest that the assumption of Racek & Harrison (1975), that endemic genera in the ancient lakes appeared independently of the cosmopolitan fauna, is invalid as far as Baikalian Lubomirskiidae is concerned.

Branch length shows that divergence of Lubomirskiidae and Spongillidae took place much later than divergence of their common ancestor. It

provides support for Efremova (1981) that Lubomirskiidae is not a relic fauna, but a flourishing group of Lake Baikal organisms. This also confirms Talievs' (1955) opinion about the relatively fast evolution of the Lake Baikal fauna. It will be interesting to check this assumption using palaeontological studies of sponge spicules in the bottom sediments of Lake Baikal.

It is possible that the scenario of Baikalian sponge fauna formation is similar to that of the Baikalian Turbellaria, which is closely related to cosmopolitan species (Timoshkin, 1995). Thus, although a part of Lake Baikal fauna really has marine origin, Baikalian sponges have a typical freshwater origin.

However, as the evolution of animal 18S rDNA is non-clock-like, it is advisable to conduct investigations into the cytochrome oxidase genes whose sequences are not yet available for Porifera. This study would allow estimates to be made of divergence times between Lubomirskiidae and Spongillidae. Our tree also demonstrated an earlier divergence of *Spongilla* from the common branch of freshwater sponges. However, the few freshwater genera yet analysed, and insufficient variability of 18S rDNA, does not yet provide any unequivocal support to hypothesise relationships between certain freshwater genera. To study relationships between closely related freshwater genera, we need data from more variable regions of the gene. Work on internal transcribed spacers (ITS1 and ITS2) is currently in progress. *Trochospongilla* is likely to be a possible direct ancestor of Lubomirskiidae. This genus has no microscleres, and spicules have maximal mutability. According to preliminary data, *Axinella, Microciona, Halichondria* and *Tetilla* are the most closely related to the present freshwater sponges. It is probable, however, that obtaining new data on the other marine sponge sequences, for example other Haplosclerida, will substantially change the scheme presented here.

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